

The University of Chicago

A MICRO-CAPON ASSAY
METHOD FOR ANDROGENS AND ITS
APPLICATION TO HUMAN
BLOOD

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY
JUNE, 1942

By
CARTER DUPUY JOHNSTON

CHICAGO, ILLINOIS

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PART I

Upon the observation made by McGee (1) in 1927, that lipid extracts of testis tissue produce measurable growth in the combs of capons, rests much of the progress made in the field of male sex hormones. The subsequent work of Gallagher and Koch (2,3) established a satisfactory assay method for these hormones and thereby contributed greatly to their successful isolation. A description of this research has no place in these pages, but reference should be made to the numerous reviews on the androgenic hormones, of which that of Koch (4) is comprehensive.

Many tests have been devised for measuring the male hormone activity of various types of preparations, but most of these methods are of qualitative value only. A few such tests are: the changes in cytology and weight of the prostate, seminal vesicles, and Cowper's gland of castrate and immature mammals; the length and weight of the penis; the survival time of spermatazoa; the maintenance of ejaculation ability; crest growth of the salamander; the production of mating coloration in the male bitterling; anal fin changes in the guppy; and the blackening of the bill of the castrated male English sparrow. Of all these criteria, and of various others, the two most practical have been the weight-increase of the prostate and seminal vesicle in the rat and mouse, and the growth of the capon's comb. The latter is more reliable and is more widely employed. For a discussion of the procedures involving the accessory sex organs of rats and mice, the reader should refer to the review by Gustavson (5) in which numerous references to the original literature are to be found.

Since the present paper deals with the application of a modification of the capon comb-growth procedure to extracts of blood, it is in order to consider briefly some of the techniques involved in the use of this reaction.

The most complete measure of comb-growth would be one which determines the increment of all three dimensions--either by means of the volume or the weight. Such methods have been described. Engle (6) devised an instrument for measuring volume by means of

water displacement; but his paper shows no diagram of the device, nor does it present data to furnish any idea of the accuracy of such a method.

A comb-weight method is used in the baby-chick assay (Burrows, Byerly, and Evans [7]; Dorfman and Greulich [8]; Frank, et al. [9,10]) in which the animals are sacrificed at the conclusion of the determination. It should be noted that this method of assay is open to the very serious objection that it is not possible to ascertain the initial weight of the test organ, and that the fluctuations in the comb weights of untreated chicks are very marked. The method has recently been criticized in detail by workers in three different laboratories (Duff and Darby [11]; McCullagh and Guillet [12]; and Hoskins, Beach, Coffman and Koch [13]) and will not be discussed further here. It is sufficient to note that the method is of value only where roughly quantitative results are desired; speed and economy of materials are its chief assets.

The next best measure of comb-growth would be some means of determining the area increase. This has been attempted by several investigators. Some have photographed the shadow of the comb and subsequently measured the area with a planimeter, or have cut out a shadow-tracing from a piece of paper and then weighed the pattern. Other workers devised means of measuring the comb-area photoelectrically. Still others used a modified planimetric procedure, eliminating the shadows cast by the barbles and measuring only the area of the trapezium thus obtained. All these methods are subject to serious error, the greatest of which is the lack of an accurate base line.

The simplest procedure, both from the standpoint of facility and brevity, is that proposed originally by Gallagher and Koch (2), in which the measure of comb-growth is taken as the increase in the length plus the height (L plus H response) of the comb. The length is the maximum distance from the point of juncture of comb and bill to the back of the comb, and the height is that of one particular barble. An ordinary millimeter ruler is used.

Standard preparations are always run in parallel with unknowns, and from the response to these doses it is possible to calculate the concentration of the androgenic material in an unknown. The results were first expressed in terms of "bird units,"

but when the International Standard for androgens was adopted in 1935, this unit of activity was used in all subsequent work.

In all the earlier work on the determination of androgens, the material to be assayed was dissolved in a suitable vehicle, such as sesame or olive oil, and injected intramuscularly or subcutaneously. Because of the large dilution which resulted before the hormone reached the site of action, relatively large amounts of a preparation were needed. The first effort to apply the hormone directly to the comb was made by Fussgaenger (14), who brushed the oily solution on the test organ and found that much smaller doses were required to produce a given response than were needed by the older injection technique. However, the application of such a solution to the head entails varying amounts of loss due to the oil spreading onto the adjacent feathers of the head, and such a solvent leaves the comb rather gummy and difficult to measure.

In 1939, Zondek and Sulman (15) reported using alcoholic solutions of androgens brushed on the combs of chicks and claimed to be able to detect as little as 0.5 of a microgram of testosterone by this procedure.

With these modifications in mind, then, the following assay method was devised.

EXPERIMENTAL

The response, as before, is expressed in terms of the average increase in the length plus the height of the combs of eight to ten birds, over that of the controls. The androgenic material is applied directly to the comb in ether solution, 0.05 cc. (measured from a calibrated pipette) per day for ten days, and the final measurement made on the eleventh day. Standards of pure androsterone are run at the same time in two levels, usually 0.02 and 0.06 microgram per capon per day. Thus it is possible to assay accurately a minimum of two micrograms total amount of androsterone. Control birds are treated in like manner with ether only.

Between applications the samples are kept in the cold room at -4°C . and are transported to and from the animal quarters in a bucket of ice water. The factor of ether evaporation has been checked repeatedly and is negligible. The samples are kept tightly stoppered at all times, except during the actual application. Ether, rather than alcohol, was chosen as the solvent since it was

noticed that the birds have a tendency to shake off some of the alcoholic solution; whereas ether evaporates so rapidly that the androgen is left entirely on the comb, and with such small volumes there is no spreading to adjacent feathers.

One additional and highly important modification has been made. As previously mentioned, the determination of the length was first made by measuring from the point of juncture of comb and bill. It was realized that considerable error was involved in this technique, since quite frequently birds injured their combs by running into the wire netting of the cages; and, as a result, accurate measurement was impossible. The new method is simply to measure from the foremost "corner" of the nostril, and this procedure has resulted in a remarkable increase in precision for this measurement.

With this standard technique in operation, it was desirable to determine the optimum conditions of assay with regard to dosage, frequency of application, duration of the assay, and relative potencies of various androgenic compounds. These factors will be discussed at some length.

Although in the older assay method the hormone preparation was injected for five days, the last measurement being made on the sixth day, the present work shows that a ten-day period of application gives better results. That is to say, a greater response is obtained for a given total dose when the hormone is administered in small amounts for a longer time, than when it is employed in larger doses for a few days. Figure 1 illustrates the point graphically. It can be seen that giving the total dose over a ten-day period produces a distinctly greater response than does a five-day application. However, application three times a day produces results no better than those obtainable with one.

As with many bio-assays, a very high dosage produces a small response increment. Thus, in one of the experiments (not shown), five micrograms total dose produced a response of about 4 mm., while ten and twenty times this amount gave an average growth of only 4.5 and 5.0 mm., respectively. This phenomenon has been reported repeatedly, as for instance, by Emmens (16), who says ". . . too high dosage fails to produce the maximal response." It should be mentioned that in order to see whether or not there is a lag in response at these very high levels, these animals were

FIVE AND TEN DAILY APPLICATIONS OF ANDROSTERONE

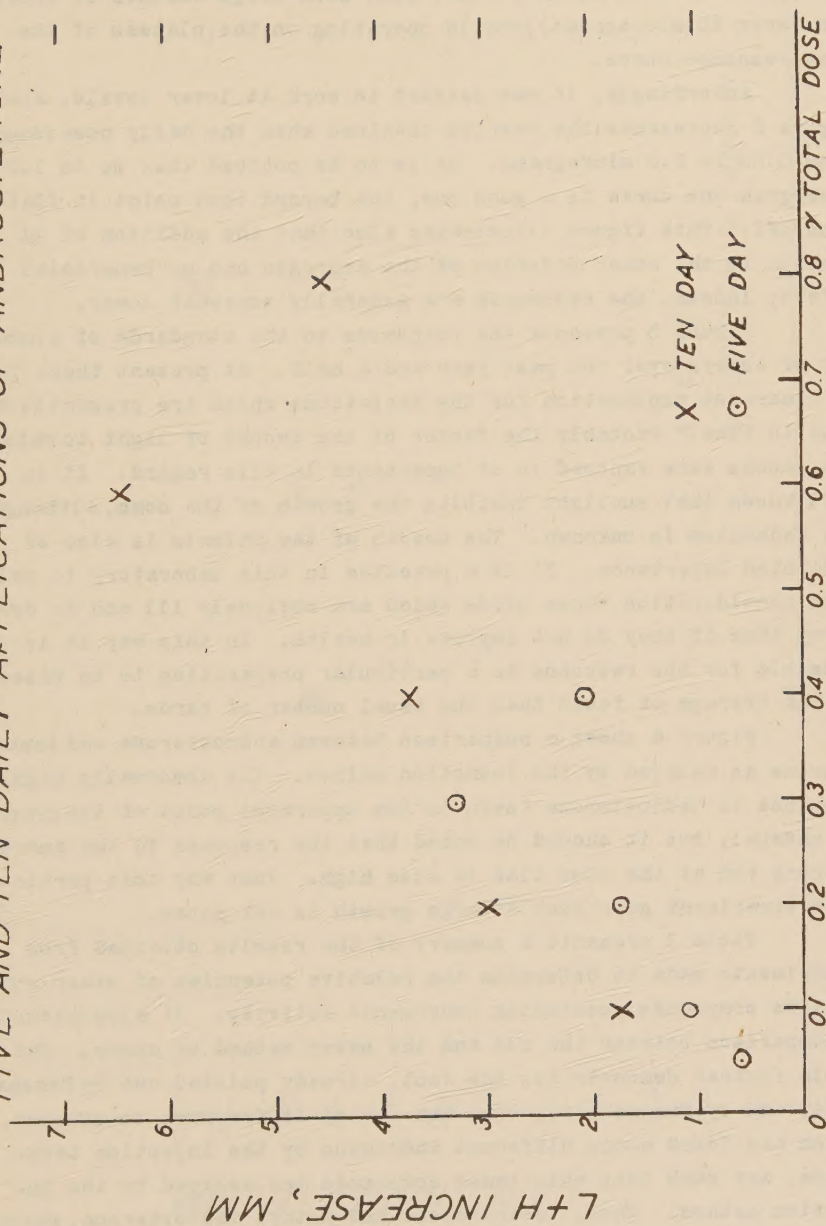


FIGURE 1

observed for a considerable time after cessation of the application. It was demonstrated satisfactorily that there is no hold-over, and the conclusion is that with such large amounts of androgen (over 20 micrograms) one is operating on the plateau of the dose-response curve.

Accordingly, it was decided to work at lower levels, and Figure 2 represents the results obtained when the daily dose ranged from 0.25 to 2.0 micrograms. It is to be noticed that up to 1.0 microgram the curve is a good one, but beyond that point it flattens off. This figure illustrates also that the addition of 1% lanolin to the ether solution of the androgen has no beneficial effect; indeed, the responses are generally somewhat lower.

Figure 3 presents the responses to the standards of a number of assays over the past year and a half. At present there is no clear-cut explanation for the variations which are present from time to time. Probably the factor of the amount of light to which the capons were exposed is of importance in this regard. It is well known that sunlight inhibits the growth of the comb, although the mechanism is unknown. The health of the animals is also of undoubted importance. It is a practice in this laboratory to omit from consideration those birds which are obviously ill and to destroy them if they do not improve in health. In this way it is possible for the response to a particular preparation to be based on the average of fewer than the usual number of birds.

Figure 4 shows a comparison between androsterone and testosterone as assayed by the inunction method. The abnormally high response to testosterone (seen in the uppermost point of the graph) is unusual, but it should be noted that the response to the androsterone run at the same time is also high. Just why this particular experiment gave such a large growth is not known.

Table 1 presents a summary of the results obtained from experiments made to determine the relative potencies of other crystalline compounds possessing androgenic activity. It also shows a comparison between the old and the newer method of assay. The table further demonstrates the fact, already pointed out by Desseau (17), and by Emmens (16), that the marked differences in potency, which are found among different androgens by the injection technique, are much less when these compounds are assayed by the inunction method. Thus, testosterone and methyl testosterone, which,

FIVE DAILY APPLICATIONS OF ANDROSTERONE

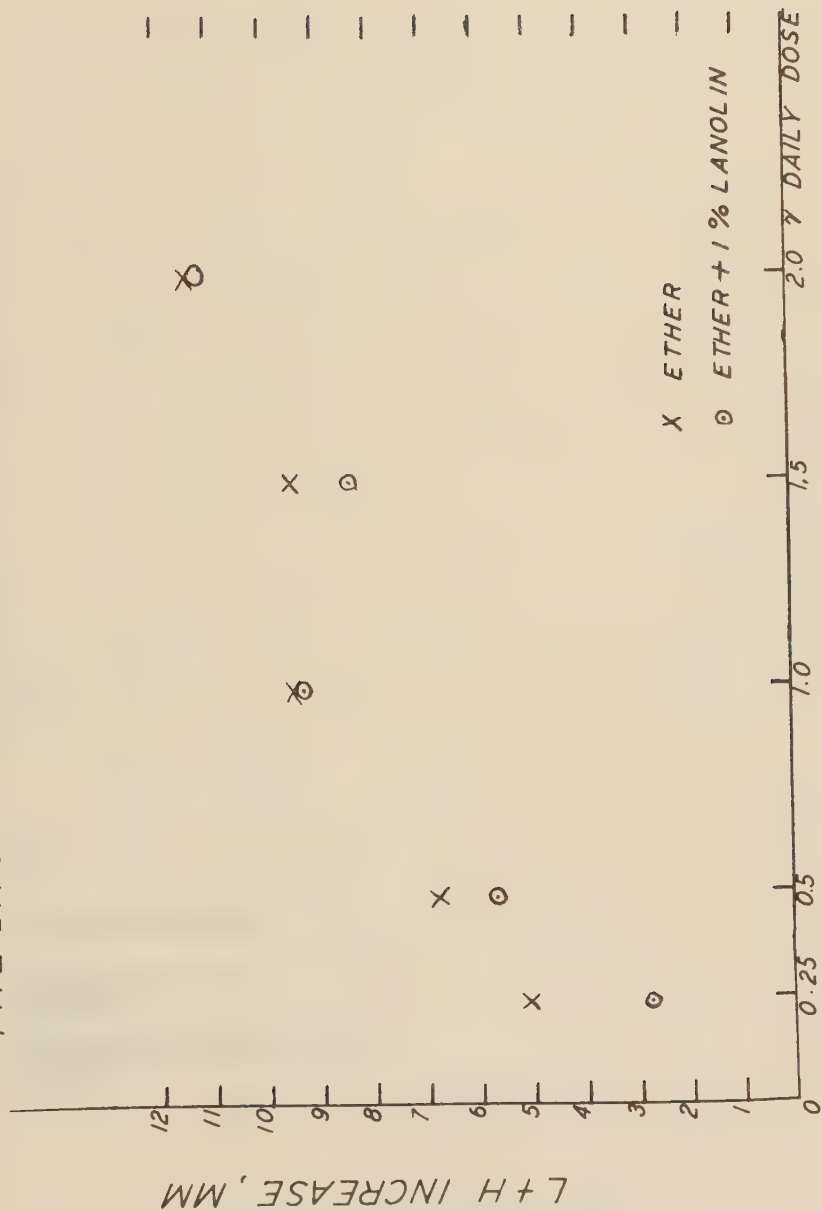


FIGURE 2

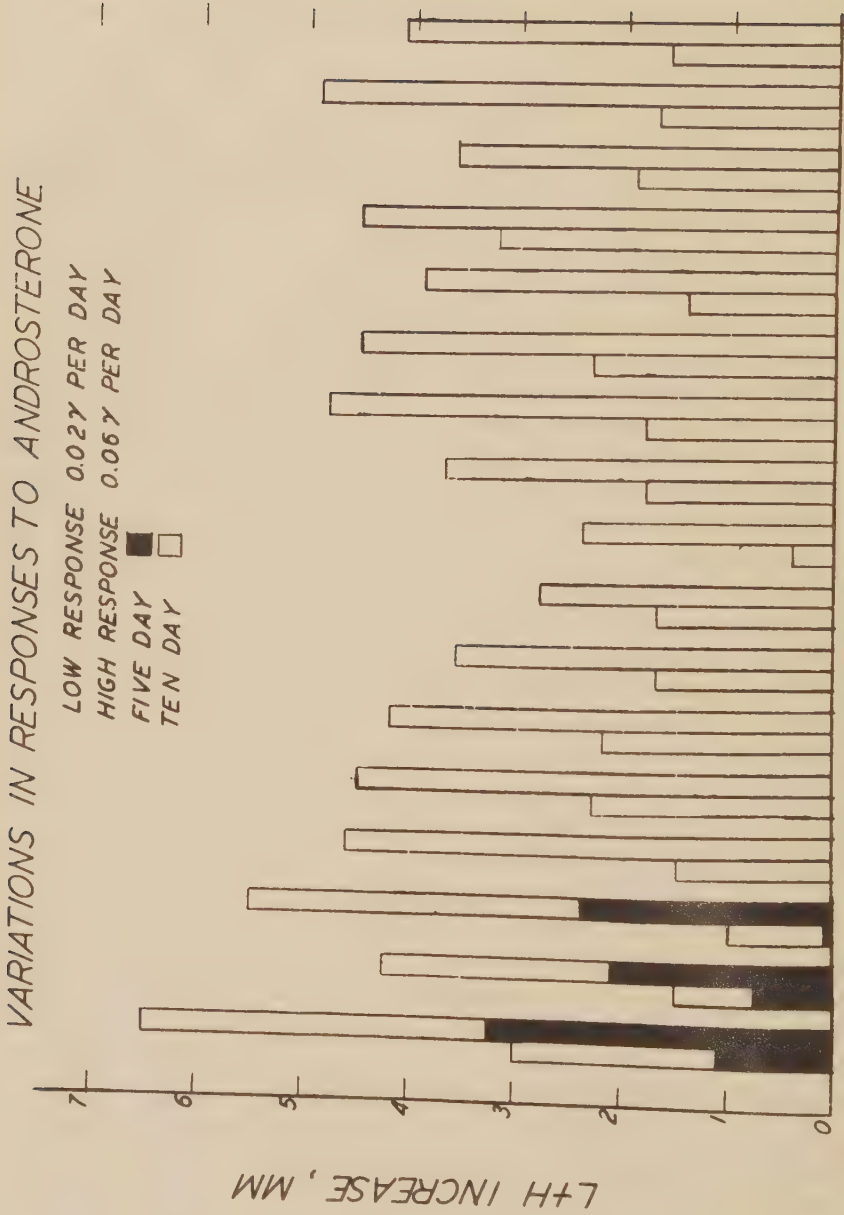


FIGURE 3

COMPARISON OF ANDROSTERONE AND TESTOSTERONE

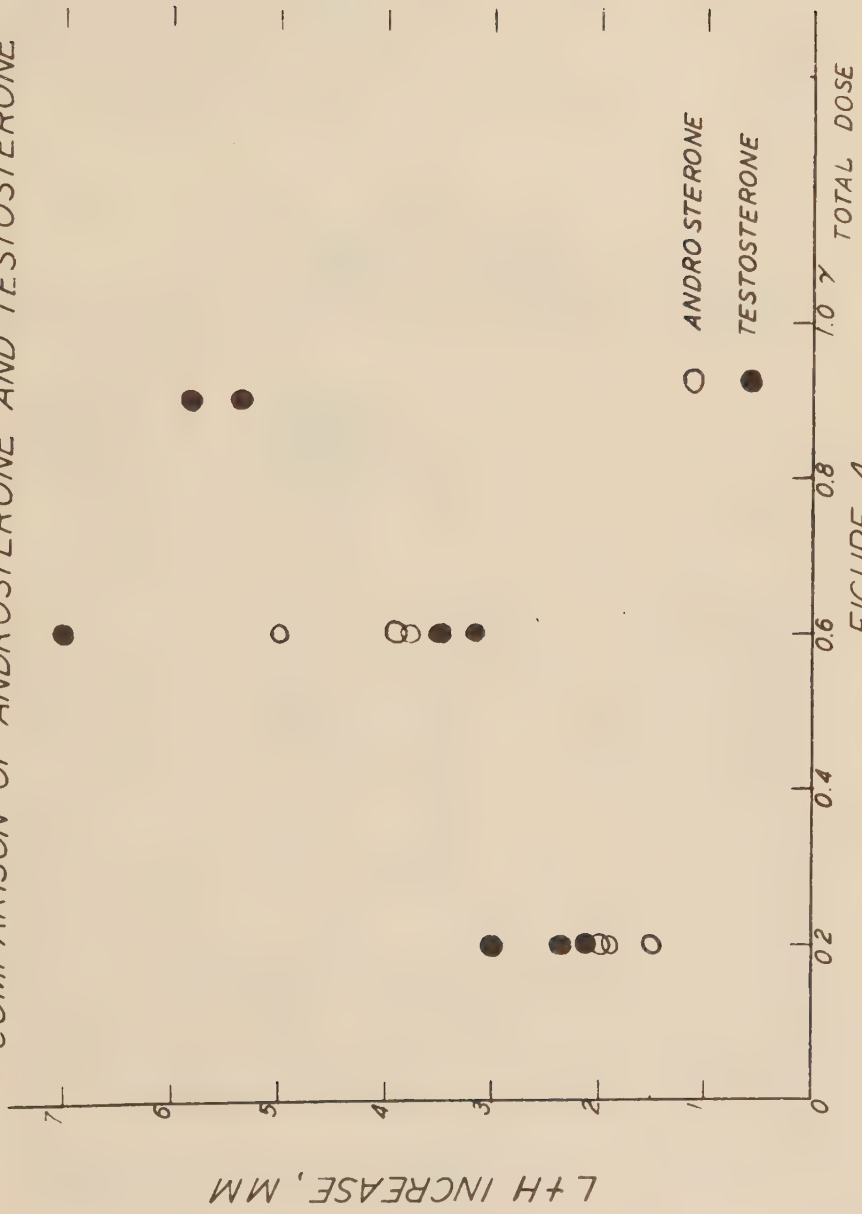


FIGURE 4

by injection, are six to seven times as active as androsterone, are, by application, of the same, or of only slightly greater, potency. Emmens (16) reports from his own data that testosterone is active to the extent of 140% (with androsterone considered 100%), as compared with our value of 90 to 100%. Voss (18) reports this compound as being 50 to 100% as active. In cases where we have investigated the same compounds as these men, our results are in agreement with theirs.

TABLE 1
RELATIVE ACTIVITIES
OF PURE ANDROGENS BY TWO CAPON METHODS

Compounds	Capon Assay Method	
	Per Cent Activity by Intramuscular Injection	Per Cent Activity by Application to Comb
Androsterone	100	100
Dehydroisoandrosterone	40	25-30
Dehydroisoandrosterone acetate	25	25-40
Dehydroisoandrosterone acetate p-carboxy phenylhydrazone..		5
Testosterone	600-700	90-150
Testosterone propionate	600-700	90-150
Testosterone propionate oxime		25
Testosterone oxime		10
Methyl testosterone	600-700	90-100

Just why the differences in potency, apparent by the injection method, are less in the newer assay technique is not clear. It is possible that photochemical reactions alter some compounds to a greater or lesser extent, and thus reduce their biological potency, but direct evidence in this regard is lacking. The nature and rate of conjugation of the various androgens may also be a factor.

It has been of encouragement to observe that comparable samples of urine extracts, when assayed by both the injection and

inunction methods, gave similar values. The figures are presented in Table 2. Although no single sample has as yet been assayed by both procedures, the fact that the samples cited are either all controls, or are from patients receiving non-androgenic hormones, and that in each series the values fall in a rather narrow range, and that this range is the same by both methods, is significant.

TABLE 2
ASSAYS ON HUMAN URINE*

Sample Number	Patient	Nature of Sample	Capon Assay Method	
			Intramuscular Injection IU/Day	Comb Application IU/Day
695	Miss F.	Pre-treatment	..	7
696	Miss F.	Growth hormone	..	7
706	Miss F.	Post-treatment	6	..
707	Miss F.	Pre-treatment	2	..
708	Miss F.	Progesterone	6	..
710	Miss F.	Progesterone	3	..
711	Miss F.	Progesterone	5	..
712	Miss F.	Post-treatment	..	4
715	Miss F.	Post-treatment	..	4
716	Miss F.	Pre-treatment	..	3
719	Miss F.	Post-treatment	..	3
689	Mr. S.	Pre-treatment	14	..
704	Mr. S.	Post-treatment	..	12

*Presented here through the courtesy of Dr. Allan T. Kenyon.

PART II

As soon as the assay procedure just described was sufficiently well established to warrant application to various types of extracts, it became of interest and importance to redetermine the male hormone titre of human blood. Due to the very small amounts present in the blood stream at any one time, accurate estimations of such material have heretofore not been feasible.

Various investigators have endeavoured to demonstrate the presence of the male principle in the blood of animals, such as bulls; and, indeed, Busquet (19) claimed that the serum of this animal, as well as that of stallions and rams, caused the male characteristics to reappear in castrate and senile cocks. He administered his material by mouth as well as by injection and claimed to produce positive results either way. Considerable doubt must be cast upon his findings, as his paper contains no actual experimental data.

Loewe, Rothschild, Rautenbusch, and Voss (20) state that an ether extract of saponified ox blood contained an active principle in an amount equivalent to one to two "mouse units" per liter of blood, when such an extract was injected into castrate mice.

Very little data is to be found in the literature regarding the male hormone content of human blood. Glaser and Haempel (21) claimed to find "large amounts" in the blood of a patient with a virilizing ovarian tumor, though they say very little was present in the tumor itself.

In 1938, McCullagh and Osborn (22) described the preparation of an extract from the blood of normal young men and reported activity amounting to 40 International Units of androgen per liter of blood. In view of the fact that they present no assay data whatever, and in light of the findings to be reported here, their figure seems fantastically high.

In his Harvey Lecture of 1937-38, Koch (23) discussed the problem of human blood-androgens and mentioned the unpublished results of experiments carried out in his laboratory, to the effect that normal human male blood contains about four International

Units of androgen per liter (expressed as androsterone). He found it necessary to inject the equivalent of 136 cc. of blood per day for five days in order to obtain a measurable response. The findings of this paper confirm his results.

It is of importance to note that the feasibility of accurate estimations of blood androgens is greatly increased by use of the inunction method, for it is obvious from the preceding section that the optimum range of dosage for capons is from about 0.01 to 0.1 microgram per capon per day. This is a much lower level than any heretofore reported, except, possibly, that claimed by Zondek and Sulman (13), and we have obtained good results administering as little as the equivalent of 0.05 cc. of blood per day to each of ten or fewer capons. Estimations easily may be made, therefore, upon 25 to 50 cc. of blood, an amount quite within the limits of practicability for clinical studies.

EXPERIMENTAL

In preliminary experiments the blood was extracted with a 3:1 mixture of ethyl alcohol and ether, the ratio of solvent to aqueous phase being 40:1. Proteins were, of course, precipitated in this extraction. The solid residue was repeatedly washed with alcohol, the washings added to the main body of the extract, and both were then evaporated to dryness on a water bath (temperature 50°C.) under reduced pressure. The dry material was then suspended in water and extracted with ether four or five times to remove free androgens. Considerable other fat-soluble matter entered the ether phase at this time.

The water fraction was then acidified (at first with 10% by volume of concentrated hydrochloric acid, but later in the work brought to a definite pH) and boiled under reflux for 15 minutes in order to hydrolyze conjugated, water-soluble forms of the androgens. The cold, acid, aqueous phase was then re-extracted with ether and this ether solution added to the first extract.

The combined ether solutions were then washed with several portions of 10% sodium hydroxide (to remove acids and phenolic compounds, such as estrogens), shaken out with water, and evaporated to dryness. If necessary, dehydration with alcohol was used at this stage. The sample was then made up to volume in alcohol, and aliquots taken for assay.

With this technique, results were not uniform; and, since it was felt that it was possible for the precipitated proteins to hold back some, if not all, androgenic material, a means was sought whereby an extract could be obtained from a solution of blood in which the solid phase was held to a minimum. It was found after several trials that, when blood was diluted to about ten times its original volume and then made alkaline with sodium hydroxide, a butyl alcohol extraction could be performed with no protein precipitation. It is known that butanol is quite efficient in removing both free and conjugated forms of androgens from urine and from testis tissue, so that it was logical to choose this solvent.

The procedure adopted was as follows: blood, diluted 1:10, was made alkaline by the addition of 7.5 grams of solid sodium hydroxide per 100 cc. of undiluted blood, and extracted repeatedly with small portions of butanol (one tenth the diluted volume). At first, ten extractions were made in this way, but two different experiments clearly showed that only five were necessary. No activity was obtained from five subsequent extractions in these instances, even when the equivalent of 0.13 cc. of blood per day was administered to the capons.

The butanol fractions were centrifuged to remove cell stroma, pooled, and evaporated to dryness as before, and the subsequent steps carried out as indicated above.

On the basis of other work being conducted in our laboratory, it was decided to operate with the aqueous phase at a definite pH during the hydrolysis. Consequently, the water solution containing the conjugated forms was brought to a pH of approximately 0.5 with sulfuric acid and boiled fifteen minutes under reflux.

It was noticed in some of the early experiments that, if the aqueous phase was neutral or alkaline, none of the deep red color (which was always present) went into the ether. Upon acidification, however, this color precipitated but was mostly soluble in ether. In order to avoid the presence of solid matter, it was thus deemed advisable to acidify the water solution prior to the first ether extraction. Most of the color returned to the water when the final ether solution was shaken out with hydroxide. Troublesome emulsions were broken by means of centrifugation.

Table 3 lists the results of several experiments made in this way. From the limited number of trials comparing ethanol-ether

with butanol as an extraction solvent (BL 113A and 114; 115 and 116), it appears that butanol is at least as good as ether, if not better. The experiments mentioned were duplicate aliquots of the same lot of blood in two separate instances. In one case, butanol removed about 2.5 times as much androgenic material as ethanol-ether.

TABLE 3
ASSAYS ON HUMAN BLOOD

Sample Number	Solvent	Androgenic Activity as IU per Liter of Blood
113A	Alcohol-ether	1.4
114	Butanol	2.3
115	Alcohol-ether	3.3
116	Butanol	8.0
118 ^a	Butanol	1.0
119 ^a	Butanol	1.5
120 ^a	Butanol	3.2
121 ^a	Butanol (first five extractions)	4.8
122 ^a	Butanol (second five extractions) ...	0
123 ^a	Butanol	2.6
124 ^b	Butanol	0.5
125 ^b	Ethanol (dried blood)	0.2
126 ^b	Butanol (dried blood)	1.0
127 ^b	Carbon tetrachloride (dried blood) ..	0

^aThese samples are aliquots of the same blood-lot.

^bThese samples are aliquots of the same blood-lot.

BL 123 was an experiment in which blood was diluted only 1:4 (25 cc. of blood made to 100 cc.), and the results indicate that this is a satisfactory procedure. Moreover, better separation between the solvent and the blood was obtained in this case.

In all these experiments aliquots were taken from pooled samples drawn from normal young men. Fifty cubic centimeters were withdrawn from each donor and received in flasks containing potassium oxalate sufficient to make the concentration of oxalate 0.2%.

Subsequent to the experiments with butanol extraction, it was decided to try to remove the androgenic material from dried blood. Accordingly, 400 cc. of pooled blood was frozen in a mixture of acetone and Dry Ice and dried by high vacuum. The material so obtained was a fine, dry powder. Aliquots by weight were taken for the extraction.

One sample each of 50 cc. blood-equivalent was treated with absolute alcohol, butanol, and carbon tetrachloride by simply shaking in a centrifuge bottle, centrifuging, and pouring off the supernatant liquid. This procedure was repeated five times, using 75 cc. of solvent each time. The extracts were beautifully clear and nearly colorless. When worked up and assayed, one extract (the carbon tetrachloride) showed no activity, while the two employing alcoholic solvents produced some response (see Table 3).

SUMMARY

The results of an investigation of the androgenic content of human male blood have been presented. The study was made possible by the development of a modified capon assay method by which it is feasible to determine with accuracy the hormone content of solutions containing as little as five micrograms of androsterone, or its equivalent. Details of the procedure are discussed and data pertinent to the assay method are presented.

The work shows that human blood contains from one to nine International Units of androgen per liter, with an average value of about four International Units.

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